

DEVELOPMENT OF AN HSV-1 GENE TRANSFER VECTOR WITH LOW TOXICITY

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Simply defined, therapeutic gene transfer involves the transfer of genetic material into the cells with the aim of correcting a disease and/or slowing down the progression of a disease. The public health significance is that therapeutic gene delivery carries the hope of curing currently incurable diseases such as cystic fibrosis and Duchenne muscular dystrophy (DMD), or providing better solutions to current treatments for conditions such as chronic pain. There is high expectation in this new generation of medicine that therapeutic gene transfer would rid the patients of the painful conditions and lead them to better lives.

One key component of this procedure is the vehicle that has the capability to deliver the therapeutic gene into different tissues efficiently and safely. A number of vectors based on retroviruses, lentiviruses, adenoviruses and adeno-associated viruses have been developed and their efficacy and safety evaluated in animal models and clinical trials.

Possessing several advantages, such as its large genome, the ability to transduce both dividing and non-dividing cells, HSV-1 based vectors represent promising candidates for gene delivery. Three types of HSV-based vectors have been developed: HSV amplicons, replication competent vectors and replication defective vectors. Replication defective vectors based on multiple

essential immediate early (IE) gene deletions carrying different transgenes have been constructed.

However, HSV vectors have disadvantages. The most prominent one is toxicity. To reduce toxicity, more viral genes are deleted in addition to the essential IE genes. One such gene is the one encoding viral regulatory protein ICP0. However, the deletion of ICP0 gene renders the vector highly inefficient in transgene expression although the toxicity is lowered. Thus, how to restrict the toxicity while retaining the expression is one of the issues that needs to be addressed in HSV vector development.

One possible solution is to lower ICP0 level through mutations in the ICP0 promoter region. To test this method, activities of reporter gene driven by mutated ICP0 promoter were assayed and the results showed that deletions in the promoter region did reduce ICP0 expression.

However, the optimal level to meet the goal could not be determined by reporter gene assays. Thus, constructs carrying mutant promoters driving ICP0 coding sequence were created. During the construction of these viruses, the positive control virus, later named JDTOZHERO, was found to carry an unexpected deletion in the 5'-UTR. This deletion gave this virus some unique features that fulfilled our goal, i.e., low toxicity and reasonable expression. This virus is characterized in detail in this thesis.

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1. Introduction

Therapeutic gene transfer has become one of the most investigated research fields in molecular medicine in recent years and has considerable potential to improve human health. The targets encompass a variety of diseases with public health significance, such as inherited disorders, cancers, arthritis, and diabetes. Therapeutic gene delivery carries the hope of curing currently incurable diseases such as cystic fibrosis and Duchenne muscular dystrophy (DMD), or providing better solutions to current treatments for conditions such as chronic pain. There is high expectation in this new generation of medicine that therapeutic gene transfer would rid the patients of the painful conditions and lead them to better lives, as well as save hundreds of thousands of dollars for the health system. According to the data from Recombinant DNA Advisory Committee (RCA), there are 695 approved protocols for human gene transfer clinical trial in the United States. Out of these protocols, 645 are for therapy, 41 are for marking and 9 are for non-therapeutic purposes. About two thirds of all the protocols (459 protocols) are for cancer treatment. Other targets include infectious diseases (mostly for HIV, 39 out of 42 protocols), monogenic diseases (60 protocols, e.g. cystic fibrosis) and other diseases/disorders (80 protocols, e.g. arthritis). The data are available online at: <http://www4.od.nih.gov/oba/rac/documents1.htm>. The concept of therapeutic gene transfer is simple: introduction of therapeutic gene into the cell and the subsequent expression of the gene product would be able to correct a genetic disorder and/or slow the progression of a disease. Great progress has been achieved through laborious efforts. Gene transfer methods have been developed and some have been tested in clinical trials

and success achieved. However, obstacles still exist, such as production of the vectors, cell targeting, expression and safety concerns. In addition, setbacks have generated disappointments along the development. The death of Jesse Gelsinger in 1999 in an adenoviral vector clinical trial and the development of leukemia-like condition after treatment with retroviral vector in two X-SCID children in France put therapeutic gene transfer under closer public scrutiny.

The currently available delivery vehicles can be categorized into two groups: The first is non-viral methods, in which naked DNA is delivered by injection, complexed with cationic lipids (liposomes), by ballistic transfer technique, or other means. Although non-viral vectors can be easily produced in large quantity and pose minimal toxicity and immunological reactions, these methods suffer from low efficiency.

The second group uses viruses as a shuttle, taking advantage of the nature that viruses are able to attach to the cell and deliver their genetic materials into the cell efficiently. The most common studied and used viral vectors today are those developed from retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV) and herpes virus. No single vector can suit all the applications. Depending on the application conditions and the desired results, such as expression level and duration, targeted tissue, different vectors may be used. Retroviral, lentiviral, adenoviral and AAV vectors are briefly described below. HSV vectors will be discussed in more details later. These vectors are summarized in the following Table 1. (For more detailed reviews, see (Fields et al. 2001; Templeton 2004; Verma and Weitzman 2005))

1.1. Retroviral Vectors

Retroviral vectors are the earliest developed vectors and were the first used in a human therapeutic gene transfer trial. Retroviruses are enveloped viruses with two copies of viral RNA

genome. The genome consists of three genes, gag, pol and env, and long terminal repeats (LTRs) at each end of the genome, which contain enhancer/promoter sequences and integration sequences. Representing retroviral vectors are vectors based on Murine leukemia virus (MuLV). The genes encoding viral polypeptides are removed in the vectors, rendering the vectors fully replication defective and leaving a 6-8 kb space for exogenous gene(s). Only the *cis*-signals essential for the vector's life cycle, including the LTRs, the packaging signal (Ψ), the primer transfer RNA (tRNA) binding site (PBS) and the polypurine tract (PPT), are retained.

Several changes in the 5' and 3' LTRs have been made to increase vector yields, improve the transgene expression and improve the bio-safety of the vector. One improvement is that the U3 region in the 5' LTR of the vector is replaced by HCMV enhancer-promoter, resulting in an HCMV-LTR hybrid vector construct. Compared to constructs with parental 5' LTR, the hybrid can greatly increase vector yields due to the strong HCMV promoter. Another improvement is the inclusion of *cis*-acting posttranscriptional regulatory element (PRE) at the 3' of the transgene. Incorporation of the PRE of woodchuck hepatitis virus (WPRE) increased the transgene expression independent of cell type or cell cycle status of the target cell.

To improve the bio-safety of the retroviral vectors, deletion mutations have been introduced in the 3' LTR. Since the U3 enhancer/promoter of the 5' LTR in the integrated provirus is derived from the 3' LTR, the deletion in U3 of 3' LTR should be carried over to 5' LTR during the reverse transcription in the target cell. These vectors are known as self-inactivating (SIN) vectors. SIN vectors eliminate the transcriptional activity in the U3 enhancer/promoter that could influence the expression of cellular oncogene close to the integration site. In addition, in some cases, heterologous promoter is desired to achieve higher or cell type specific transgene

expression. SIN deletions also remove the possibility of interference of the wild type LTR to the internal promoter.

Since the vector constructs are deleted of all the genes, the proteins required for packaging are provided *in trans*. First generation packaging cell line contains a mutant MuLV virus with deletion in the packaging signal. One safety issue with early generation packaging systems was the contamination of the replication-competent retrovirus (RCR). Further modifications have been introduced to address this issue. For example, the *att* sequence at the 5' end of the 5' LTR is removed in the packaging system to prevent integration of the helper-virus even if the helper-virus infects the target cells. An additional approach is splitting the packaging construct, with gag/pol genes on one plasmid and env gene on another. By this method, the possibility of homologous recombination is greatly reduced. Another advantage of the split packaging system is that it is easy to replace the env gene with that of other viruses (this process known as pseudotyping) to change/expand tropism and/or facilitate purification by ultracentrifugation (e.g. pseudotyping with Vesicular stomatitis virus G (VSV-G) protein).

The most concerning safety issue of retroviral vectors is insertional mutagenesis. Since retroviruses integrate into host genome after infection, there is possibility, though rare, that such insertion occurs close to a cellular proto-oncogene and drives inappropriate expression from LTR, or disrupts a tumor suppressor gene. The inappropriate activity of LTR can be addressed by SIN vectors but there is still no solution to the latter problem. The cases in France were thought be caused by insertional tumorigenesis (for detail, see [FDA briefing](#)).

1.2. Lentiviral Vectors

One of the major problems for MuLV vectors is that they are only able to infect dividing cells. On the other hand, lentiviruses, belonging to the same Retroviridae family as MuLV, have the capability to infect non-dividing, terminally differentiated mammalian cell. However, the strategies and techniques in development of lentiviral vectors are based on and similar to those for simple retroviral vectors.

The most common lentiviral vectors are based on human immunodeficiency virus type 1 (HIV-1). Lentiviruses share similar virion structure and genome organization with simple retroviruses like MuLV but the genome encodes additional regulatory proteins. Lentiviral vectors, like MuLV retroviral vectors, are constructed by deleting all the genes encoding viral proteins, thus rendering the vectors fully replication defective. The proteins are provided *in trans* by packaging/producer cells. The vector construct only contains the transgene flanked by LTRs and *cis*-signals for packaging, reverse-transcription and polyadenylation.

Like MuLV vector, CMV/LTR hybrid constructs have been created to increase the vector yields. Combined with this, deletions in U3 of 3' LTR have been incorporated into the constructs to make a SIN vector. Heterologous promoter, such as HCMV, can be used to drive the transgene expression. An alternative is to use tissue specific promoter or inducible promoter (e.g. TetOff).

Along with the development of the vector construct, several generations of packaging systems have been developed to reduce the occurrence of recombinant replication-competent virions and contamination of helper-viruses. The measures, similar to those in MuLV vector packaging cells, include deleting packaging signal in helper virus, deleting genes encodes accessory genes (*vif*, *vpr*, *vpu*, *nef*), and splitting packaging plasmid. Lentiviral vectors can also be pseudotyped with another surface protein just like in MuLV vectors.

The major safety concern of HIV-based lentiviral vector, same as in MuLV vectors, is insertional mutagenesis. In addition, since HIV is a notorious human pathogen, the effects of the regulatory proteins present in the vector virions on the target cells are being evaluated.

1.3. Adenoviral Vector

Adenovirus is a medium-sized, non-enveloped virus with a 36 kb, double stranded DNA genome which is flanked by two inverted terminal repeats (ITRs). Of the over 50 known human adenovirus serotypes, most of the vectors are derived from serotype 5 (Ad5) and 2 (Ad2). First generation vectors take advantage of the fact that E1A is essential for viral replication and expression of other viral proteins. Deletion of E1 region renders the virus replication defective. The vector can be propagated on 293 cells, which expresses E1A. Another finding is that the *cis*-acting packaging signal is required for encapsidation of the viral genome. Several strategies have been designed to insert foreign genes into the viral genome. The most widely used method relies on homologous recombination. The circular viral genome, as a plasmid, can be propagated in bacteria and the transgene on the shuttle plasmid is flanked by sequences homologous to the region of the insertion (i.e. E1 region). Co-transfecting producer cells with two plasmids with homologous regions can generate infectious virions by homologous recombination.

The most prominent problem with the first generation vectors is the generation of replication competent adenoviruses (RCA). In the second generation of Ad vectors, this problem is addressed by deleting more viral genes, such as E2, E3 and/or E4. The additional deletion not only helps reduce the chance of homologous recombination, but also eliminates potential immunogenicity from these viral products as well as makes more space for transgene. Deletion in E2 was initially hampered because of its toxicity to the producer cell until the introduction of

an inducible expression system. Deleting E4 resulted in significant loss in vector yields and this problem was addressed by either partial deletion or insertion of spacer sequence. Inducible expression systems of E4 in producer cells have also been established to eliminate the toxicity of the gene.

The third generation has been termed high-capacity, helper-dependent Ad vectors. An important development in third generation vectors is the introduction of Cre/loxP system. One loxP site is inserted in the packaging signal-deleted Ad genomic plasmid. The other loxP and the packaging signal are incorporated into the vector construct through homologous recombination from the shuttle plasmid. When the vector construct is propagated on Cre-recombinase expressing 293 cells, the region flanked by the two loxP sites is excised. The resulting “gutless” vector has almost all the viral genes deleted and only contains the ITR, the packaging signal, the viral E4 region, the transgene and the internal promoter and has an enlarged insertion capacity, up to 35 kb. These vectors require a helper-virus to propagate, thus posing a great problem for purification. An alternative strategy is to target the packaging signal in the helper virus using Cre/loxP system.

One obstacle when using Ad vectors is that Ad vectors induce strong immune response. The immune response is against both the vector and the transgene and results in the clearance of the vector and short-term transgene expression. Since Ad genome is not integrated in the host genome, re-dosing sometimes is necessary. However, immunity against the vector would prevent the re-dosing. One way to go around this problem is to use a vector from different serotype group. On the other hand, inducing immune response might be useful in vaccine development.

1.4. AAV Vectors

Adeno-associated virus is a small, non-enveloped DNA virus belonging to the parvovirus family. Clinically, AAV is not associated any known disease by itself, which makes it an attractive candidate for gene transfer vector. Of the eight known serotypes of AAV, type 2 (AAV2) is the best characterized and most of the AAV vectors developed so far are derived from AAV2. Replication of AAV depends on the presence of viral proteins from helper viruses such as adenovirus or herpes virus. In the absence of the helper virus, AAV would go into a latent infection. In latent infection with wild type AAV, the genome integrates in the specific region called AAV-S1 on human chromosome 19.

The genome of AAV contains a single stranded (ss) DNA unique region with a length of 4.4 kb flanked by two inverted terminal repeats (ITRs). *cis* sequences in ITRs are essential for genome encapsidation, genome integration and rescue, and second strand synthesis (metabolic activation). ITRs and adjacent 45 nucleotides in the unique region are the only viral structure necessary for the vector construct. In the construct, the two viral ORFs, which encode the four regulatory Rep proteins (Rep78/68 and Rep52/40) and the capsid proteins (VP1, 2, and 3), are replaced by the transgene and its promoter.

The principle of producing/packaging for AAV vectors is the same as for retroviral or adenoviral vectors: the vector construct contains the *cis*-signals and all the viral proteins are provided *in trans*. Since replication of AAV requires helper-virus functions, the producer/packaging system consists of three parts: the vector construct (which contains the *cis* signals and transgene expression cassette), the packaging plasmid providing Rep and Cap, and the construct providing helper-virus functions.

First generation producer/packaging system used adenovirus-infected cells. When the vector plasmid and packaging plasmid are co-transfected into the producer cell, the AAV genome in the

construct plasmid is excised, followed by its replication and encapsidation. Although high-titer AAV vectors can be produced by this system, wild type adenovirus contaminates these vector stocks. Thus, this system is not suitable for clinical use.

Further studies revealed that the only genes required for AAV replication from the adenovirus are E1A, E1B, E2A, E4, and viral-associated (VA) RNA genes. Based on these findings, helper-virus free system has been established. A plasmid containing the E1B, E2A, E4 and VA RNA genes is used instead of the adenovirus. The E1A function is provided by 293 cells. This system not only removes the co-production of the adenovirus but also increases the yield of the AAV vector. This phenomenon is thought to be the result of the lack of competition for replication machinery in the cell and the removal of the cell lysis caused by the helper-virus.

Improvement has also been made in the packaging plasmid. In the second generation packaging system, the ATG translation start codon for Rep is substituted with an inefficient ACG codon to attenuate the synthesis of Rep68/78 since Rep causes paradoxical inhibition on vector production if the Rep68/78 is overexpressed. Another improvement is the inserting of an addition of p5 enhancer/promoter at the 3' end of the cap gene in the packaging plasmid. By this system, high titer of rAAV vector production has been achieved.

However, problem persists in this system, i.e., the production of wild type AAV or pseudotyped wild type virus particle. This occurs through both homologous and non-homologous recombination. To address this issue, several approaches have been proposed. One method is by deleting the distal 10 nucleotides in the D sequence in the ITRs. Alternatively, spliced plasmid (Rep and Cap on separate plasmid) can be used.

One disadvantage of AAV-based vectors is the limited space provided by the vector. The optimal packaging size is between 4.1kb and 4.9kb. Although up to 5.2kb (including the ITRs) can be

packaged into the capsid, the efficiency is greatly reduced. The solution to this problem is based on the observation that the episomal genome can form head-to-tail concatemers. After co-infection of the target cells, the genomes from the two rAAV vectors form the concatemer through the homologous sequence in ITRs, thus re-joining the split expression cassette on two vectors into one continuous DNA molecule. Both *cis*-activation and *trans*-splicing models of dual vector systems have been described and showed remarkable efficiency (although lower than single vector).

In *cis*-activation model, one vector carries a strong enhancer for transcription and the other vector carries the transgene and its promoter. Recombination following infection results in the placement of the enhancer in *cis* with the expression cassette. This method has been shown effective with the SV40/CMV enhancer and luciferase gene, or lacZ gene and human elongation factor EIF α promoter/enhancer.

In the *trans*-splicing method, one vector carries the promoter and 5' part of a gene and the other vector carries 3' part of the gene. By careful arrangement of splicing donor and acceptor site, expression of the appropriate mRNA can be achieved from the read-through transcript from the concatemer. The internal ITR does not appear to interfere the read-through and splicing. Systems using this method have been shown to be effective in the expression of the transgenes like erythropoietin and lacZ.

Table 1: Common gene transfer vectors.

	Non-viral	Retroviral	Lentiviral	Ad	AAV	HSV
genome	plasmid	RNA	RNA	DNA	DNA (ss)	DNA
genome status	episomal	integral	integral	episomal	integral/episomal	episomal
insertion size	unlimited	6-8 kb	~11 kb	8 kb	5 kb	30-40 kb
infection	Dividing/non-dividing cells	Dividing cells	Dividing/non-dividing cells	Dividing/non-dividing cells	Dividing/non-dividing cells	Dividing/non-dividing cells
expression	transient	long-term	long-term	transient	long-term	transient/long-term
titer	high	medium-high	medium-high	high	high	medium-high
cytotoxicity	low	low	low	yes	no	yes
immunogenicity	minimal	minimal	minimal	strong	low	low

2. Herpes Simplex Virus

2.1. General Virology of Herpes Viruses

Herpesviruses are widely found in the nature, infecting a vast variety of vertebrates as well as some invertebrates. Typically, herpesviruses are classified into three subfamilies: alphaherpesviruses (e.g. HSV-1 and HSV-2), betaherpesviruses (e.g. cytomegalovirus) and gammaherpesviruses (e.g. Epstein-Barr virus) based on tissue tropism, pathogenicity and behavior in cell cultures.

Virions of all herpes virus family members share common features: a core containing linear, double-stranded DNA; an icosahedral capsid consisting of 162 capsomers; an amorphous layer called tegument between the capsid and the envelop and an lipid envelop with integral viral encoded glycoproteins.

All viruses in this family share some biological properties: a) All herpesviruses encode a large array of proteins involved in nucleic acid metabolism (e.g. thymidine kinase, ribonucleotide reductase), DNA synthesis (e.g. DNA polymerase) and protein processing (e.g. protein kinase). b) The synthesis of the viral genomes and capsids occurs in the nucleus and the capsids are enveloped as they pass through the nucleic membrane. c) Production of progeny virions inevitably results in the lysis of the cell. d) All herpesviruses possess the ability to establish a latent infection state in their natural host and latent genome is capable to replicate upon reactivation.

HSV-1, along with HSV-2, varicella-zoster virus (VZV), bovine herpes virus 1 (BHV-1), equine herpes virus 1 and pseudorabies virus (PRV), is classified into alphaherpesvirus. They show a

variable host range but replicate fast and spread rapidly in cell cultures resulting in complete cell destruction. Surprisingly, all these viruses have the ability to establish latency in the hosts. The primary latency sites are, but not limited to, nervous tissues: sensory and autonomic nerve ganglia and the central nervous system (CNS). (Fields et al. 2001)

2.2. The Virion

The HSV virion consists of four components: a) a DNA-containing, electron opaque core, b) an icosahedral capsid surrounding the core, c) an amorphous tegument layer surrounding the capsid and d) lipid envelop with embedded viral encoded glycoproteins.

There are at least 30 proteins contained in the virions. Of these proteins, a large group are glycosylated and present on the envelope. These glycoproteins are gB (VP7 and VP8.5), gC (VP8), gD (VP17 and VP18) and gE (VP12.3 and VP12.6), gG, gH, gI, gK, gL, and gM. Glycoproteins form spikes on the virion surface and are not evenly distributed. They are responsible for viral attachment, entry and cell-to-cell spread.

The tegument between envelope and the capsid contains some important regulatory proteins. The most notable ones are VP16 (aka α -trans-inducing factor, α TIF) and virion host shut-off (vhs, UL41). (Fields et al. 2001)

2.3. HSV-1 Genome Organization

The HSV-1 genome is a linear, double-stranded DNA approximately 152 kb in length with high GC content (68%). The genome can be viewed as consisting of two covalently linked components, L (long) and S (short). L component contains a 108 kb unique sequence (U_L) flanked by inverted repeat sequence of about 9 kb (termed TRL and IRL). S component

possesses a 13 kb unique sequence (U_S), bracketed by a different inverted repeat sequence of about 6.6 kb (IRS and TRS). A short (200-500 bp) repeating sequence, called “a” sequence, is found at both ends and at the IRL/IRS junction. The “a” sequence contains important signals for genome circularization and packaging. Some of the viral genes are encoded in the repeat sequences thus there are two copies present in one genome, e.g. ICP0 and ICP4. There are three origins of replication, one is located in the middle of the U_L sequence (oriL) and two are located in repeating sequences flanking U_S . oriL and at least one of the oriS are dispensable. The HSV-1 genome encodes more than 80 open reading frames (ORFs), of which almost half are not essential for viral growth under cell culture conditions. (Fields et al. 2001)

2.4. Lytic and Latent Infections

2.4.1. Lytic Life Cycle

Epithelial tissues are the common primary infection sites of HSV-1. Upon contact with the cell, the virion attaches to the cell, initially by non-specific binding by gC to cell surface heparan sulphate. Specific bindings then occur between viral glycoproteins and cellular receptors (e.g. hveA and hveC), and this triggers the fusion of the virion envelop with plasma membrane. This process requires multiple viral glycoproteins (e.g. gB, gD and gH/gL). The capsid is released into the cytoplasm and transported to the nuclear pores and viral genome is subsequently released into the nucleus. The expression of viral gene products begins with the initiation of immediate early (IE) gene expression in the presence of the tegument protein VP16, which starts a temporal coordinated expression cascade. After viral genome replication and expression of structural proteins, the genome is packaged into the newly synthesized capsid and the virion gets its envelope when traveling across the nuclear membrane. The viral particle then travels through the

cytoplasm and egresses, most likely via the Golgi apparatus. The released virions can start a new round of lytic infection or establish latency after retrograde transport to neurons in the sensory ganglia. (Fields et al. 2001)

2.4.2. Temporal Expression of HSV-1 Genes

The HSV-1 genome encodes more than 80 ORFs and the expression of these genes is coordinately regulated and timely ordered. Based on the expression kinetics, the viral genes can be divided into three major groups: immediate early (IE, or α), early (E or β) and late (L or γ).

There are five IE genes, which encode ICP0 (RL2), ICP4 (RS1), ICP22 (US1), ICP27 (UL54) and ICP47 (US12). Originally, IE genes are defined as genes whose expression occurs in the absence of *de novo* protein synthesis. The synthesis of IE proteins reaches peak rate at about 2-4 hours post infection. The transcriptions of IE genes are initiated by the tegument protein VP16. VP16 recognizes a complex of cellular components Oct-1 and HCF and the *cis*-acting regulation element GYATGNTAATGARATTCYTTGNGGG (often designated as TAATGARAT for the most conserved part) present in one to several copies in the IE gene promoters.

IE proteins ICP0, ICP4, ICP22 and ICP27 all play critical roles in viral gene expression and their functions will be discussed in later sections. ICP47 does not affect transcription but rather blocks the loading MHC class I molecules with antigenic peptides by interference with TAP.

The expression of E genes requires IE gene products but not viral DNA synthesis. Most of the E proteins are involved in nucleic acid metabolism (e.g. thymidine kinase) and DNA synthesis (e.g. DNA polymerase). Following E genes expression, the viral DNA synthesis begins.

Most L genes encode structural components of the viral particle. They can be further divided into two groups: L1 (often referred as leaky-late) and L2 (true late). L1 genes are expressed to certain

degree in the absence of DNA replication but require DNA synthesis for maximal expression. On the other hand, no appreciable accumulation of L2 gene products can be detected in the absence of DNA synthesis. (Fields et al. 2001)

2.4.3. Fate of the Infected Cells

Upon infection, cells undergo major biochemical and structural changes. With the exception of post-mitotic sensory neurons, HSV-1 infection ultimately leads to the complete destruction of the cells.

Immediately following the entry of the virus, host DNA synthesis is shut off. Cellular gene expression is turned off by the virion host shutoff (vhs) protein (UL41), which destabilizes both cellular and viral mRNA.

In addition, cellular structural changes occur along with the expression of viral proteins. For example, ND10 are dispersed by ICP0; viral glycoproteins are inserted into the membranes. (Fields et al. 2001)

2.4.4. Latent Infection

Virtually all herpesviruses have the capability of establishing and maintaining latency in their host for its lifetime and reactivating from time to time. Following initial lytic infection at the epithelial tissues, the newly synthesized virions are taken up by the termini of the sensory nerves innervating the primary infection site. Virions are carried to the neuron bodies in the sensory ganglia by retrograde axonal transport. Exactly how the virions are transported is not clear yet, nor is whether the tegument components are transported along. Initially, infected neurons support viral replication. However, after a few days, no free virus can be detected within ganglia and

latency has been established. The viral genome is sequestered in the neuron nucleus with all expression switched off except one set of transcripts known as latency-associated transcripts (LATs). The genome is kept in a non-replicating state probably as a circular episome. Upon certain stimuli that usually cause stress to the host or the neurons, such as UV radiation, hyperthermic shock, ganglia explantation, the dormant genome can reactivate and produce progeny virions. These progeny virions are transported along the axon to the initial infection sites and cause recurrent infections. (Fields et al. 2001)

2.4.5. Establishing Latency

The “decision” of entry of latency is made early in the infection but the exact initial molecular event is unclear. Viral DNA replication is not a prerequisite since HSV mutants incapable of replication are capable of establishing latency in neurons. It seems that no single viral gene is required to establish latency as well. Several hypotheses have been proposed but all have some limitations. The most obvious one is a block at the IE stage since some IE products are required for the expression of E and L genes and the absence of these IE genes products suppresses global viral expression. There are several propositions how IE block is achieved in neurons. One suggests the cellular component Oct-1, required by the VP16 transactivation, is lacking in neurons. Another suggests Oct-2, expressed in neurons, competes for the binding sites in the Oct-1-HCF-TAATGARAT complex and thus inhibit the function of Oct-1. Some evidence suggests neuron-specific differences in the activity of HCF could contribute to the IE suppression. LATs might also repress IE expression. (Fields et al. 2001)

2.4.6. Genome during Latency

The genome remains in a non-replicating state, most likely as a circular episome. The genome is believed to have a structure similar to that of the heterochromatin, bound by histones. The genome copy number in latently infected ganglia neurons ranges from one to thousands.

All gene expression from the genome is switched off with the exception of LATs. LATs are a set of transcripts accumulating to high level during latency. The genes for LATs are located in the terminal repeats flanking U_L. The major products are 2 kb and 1.5 kb RNAs, predominantly localized to the neuronal nucleus. These transcripts are anti-sense to and partially complementary to the coding sequence of ICP0 and thought to be stable introns cleaved from a longer, 8.5 kb precursor. The function of LATs is not clear. LATs are not required for either replication nor establishing and maintaining latency. (Fields et al. 2001)

2.4.7. Reactivation

One unique feature of the latent genome is that it is readily to reactivate. The stimuli include UV radiation, heat shock and explantation of ganglia. The wide range of stimuli generally is linked to cause stress in the host or the neurons. Some drugs can also initiate reactivation in cultured neurons. The signal transduction that leads to reactivation remains unclear. (Fields et al. 2001; Danaher et al. 2003; Miller et al. 2003)

2.5. Functions of IE proteins ICP4, ICP22 and ICP27

ICP4 is the major viral transcription regulator. It is essential for viral replication in cell culture and is required for the activation of both early and late genes. It also represses the expression of

IE genes (including its own expression). The transactivation is thought involving TFIID through TBP-associated factor (TAF) 250 and the repression involves TFIIB.

ICP27, another essential gene product, is involved in both transcriptional and post-transcriptional regulations in the transition from early to late gene expression. Its functions include polyadenylation site selection and transportation of intronless viral RNA out of nucleus. ICP27 also inhibits mRNA splicing and thus gives the virus an edge over the cellular defense (only four viral genes are spliced and three of the four genes are IE genes).

ICP22 is responsible for an altered phosphorylation state of RNA pol II. It is necessary for efficient viral replication and expression of ICP0 and a subset of late genes in certain rodent cell lines and primary human cell. (Fields et al. 2001)

2.6. Functions of ICP0

ICP0 is a 775-amino acid, RING finger protein. It is not essential for viral growth in cell cultures but viral replication is greatly impaired when ICP0 is inactivated, especially at low multiplicity of infection (MOI) (Stow and Stow 1986). The exact function of ICP0 is not fully understood yet. Several lines of evidence suggest ICP0 is a multiple functional protein (Davido et al. 2003). Apparently, it functions as a potent transactivator, being able to activate a wide range of promoters introduced into the cell by infection or transfection. However, unlike most transactivators, ICP0 does not bind DNA or known transcription factors with the exception of BMAL1. (Advani et al. 2001; Davido et al. 2002; Davido et al. 2003). Other evidence suggests that it function as an E3 ubiquitin ligase (Boutell et al. 2002). It has been shown that ICP0 encodes two distinct E3 ubiquitin ligase activity domains, herpesvirus ubiquitin ligase 1 (HUL-1) in exon 3 and HUL-2 mapped to the RING finger domain in exon 2 (Everett 2000; Parkinson and

Everett 2001; Van Sant et al. 2001; Boutell et al. 2002; Hagglund and Roizman 2002; Hagglund et al. 2002; Hagglund and Roizman 2003). ICP0 dissociates nuclear structure ND10 (also known as PML nuclear body or PODs) at early stage of lytic infection, probably by degradation of the main component proteins PML and Sp100 through HUL-2-proteasome pathway (Gu and Roizman 2003).

It has been suggested ICP0 functions through several ways to disarm innate immunity. a) By degrading PML, ICP0 disrupts interferon-induced antiviral responses (Chee et al. 2003). b) ICP0 represses IFN-independent antiviral mechanism by repressing the IRF3 pathway. c) By dissociate ND10 (Carbone et al. 2002) and DNA-dependent protein kinase (DNA-PK) catalytic subunit (Parkinson et al. 1999), ICP0 disrupts cellular double strand break repair system, which may silence the viral transcription. Thus, the function of ICP0 mimics that of a “de-repressor” of the cellular repression mechanism (Jackson and DeLuca 2003). These are in agreement with the fact that ICP0 is vital in transition from lytic to latent infection and that the effect of ICP0 deficiency on virus growth is greater at low multiplicity of infection (MOI) than at high MOI.

ICP0 has also been shown to be of importance during establishing latency and during reactivation. ICP0-defective viruses established latency about 1000-fold less efficiently than a wild type virus, and the defect could be overcome by co-infection with an ICP0-expressing adenovirus. ICP0-deficient virus could reactivate, but reactivates poorly. The exact role of ICP0 during reactivation is still unclear (Preston 2000).

In some tumor-originated cell lines, viral replication appeared to be ICP0-independent, high MOI or low. One example is human osteosarcoma cell line U2OS. U2OS cells appear not to be able to suppress viral gene expression in the absence of ICP0 as seen in cell lines such as Vero. Transgene expression from a viral construct could be achieved independent of ICP0. The

mechanism underlying is not clear yet but this phenomenon is the basis to titration viruses using transducing units.

3. HSV-1 as a Gene Transfer Vector

As a gene transfer vector, HSV-1 possesses several advantages: its large genome can accommodate large and/or multiple genes; it is able to transduce a variety of dividing and non-dividing cell types; the genome does not integrate into the host genome; it can establish lifetime latency in sensory neurons; it is able to evade immunity surveillance efficiently. It also has some drawbacks: some of the viral gene products are quite toxic, and the broad range of cell types it can transduce also means non-cell-type specific targeting. (Burton et al. 2001; Burton et al. 2002; Burton et al. 2003; Goins et al. 2004)

Currently, there are three widely studied types of HSV-1 vectors: replication-competent vectors, HSV-1 amplicons and replication-defective vectors.

Replication competent vectors are developed solely for cancer treatment. These oncolytic viruses are designed to be able to replicate in tumor tissues but not in normal tissues. This is achieved by deleting certain viral gene such as the viral thymidine kinase (TK) gene (UL23), neurotoxic factor γ 34.5 and the large subunit of the HSV ribonucleotide reductase (RR) gene (UL39) singly or in combination.

Amplicons are plasmids that contain the viral replication origin, cleavage/packaging sequence and bacterial replication origin. Cloning is performed in bacteria and the amplicon is packaged by helper-virus or by helper-virus-free system. It does not contain any viral genes thus would not express any viral protein and thus would pose minimal toxicity and immunogenicity. It has been shown that HSV-amplicons could support long-term exogenous gene expression. However, this

system still suffers from problems such as low titer, low stability and contamination of helper-virus if the helper-virus packaging system is used.

Replication defective vectors are based on viruses with deletions in essential genes. Deletion in one or both of the two essential IE genes, ICP4 and ICP27, will disable the replication of the virus. Replication defective viruses must grow on cells that provide the inactivated essential gene product(s) *in trans*. (Fields et al. 2001; Templeton 2004)

3.1. HSV-1 Vectors with Multiple IE Gene Deletions

As mentioned above, HSV-1 genes are expressed in a temporally organized cascade manner during the acute infection, disrupting the single essential IE gene ICP4 will limit the viral gene expression to only the remaining IE genes and some early and late genes. With an additional removal of another IE gene ICP27, the expression of most early and late genes is eliminated. In addition, IE genes, with the exception of ICP47, are individually toxic to most cell types when expressed in high levels (Johnson et al. 1992; Johnson et al. 1994). Thus, deletions in IE genes not only eliminate the toxicity associated with the IE genes, but also eliminate the possible toxicity associated with the early and late genes.

Vectors with multiple IE gene deletions have been constructed in this lab and by others. Compared to a viral vector only deficient for ICP4, toxicity of a vector deleted for ICP4, ICP22 and ICP27 is greatly reduced in cultured Vero cells (Wolfe et al. 2004). A virus deleted for ICP0, ICP4, ICP22 and ICP27 is essentially non-toxic, even at high MOI.

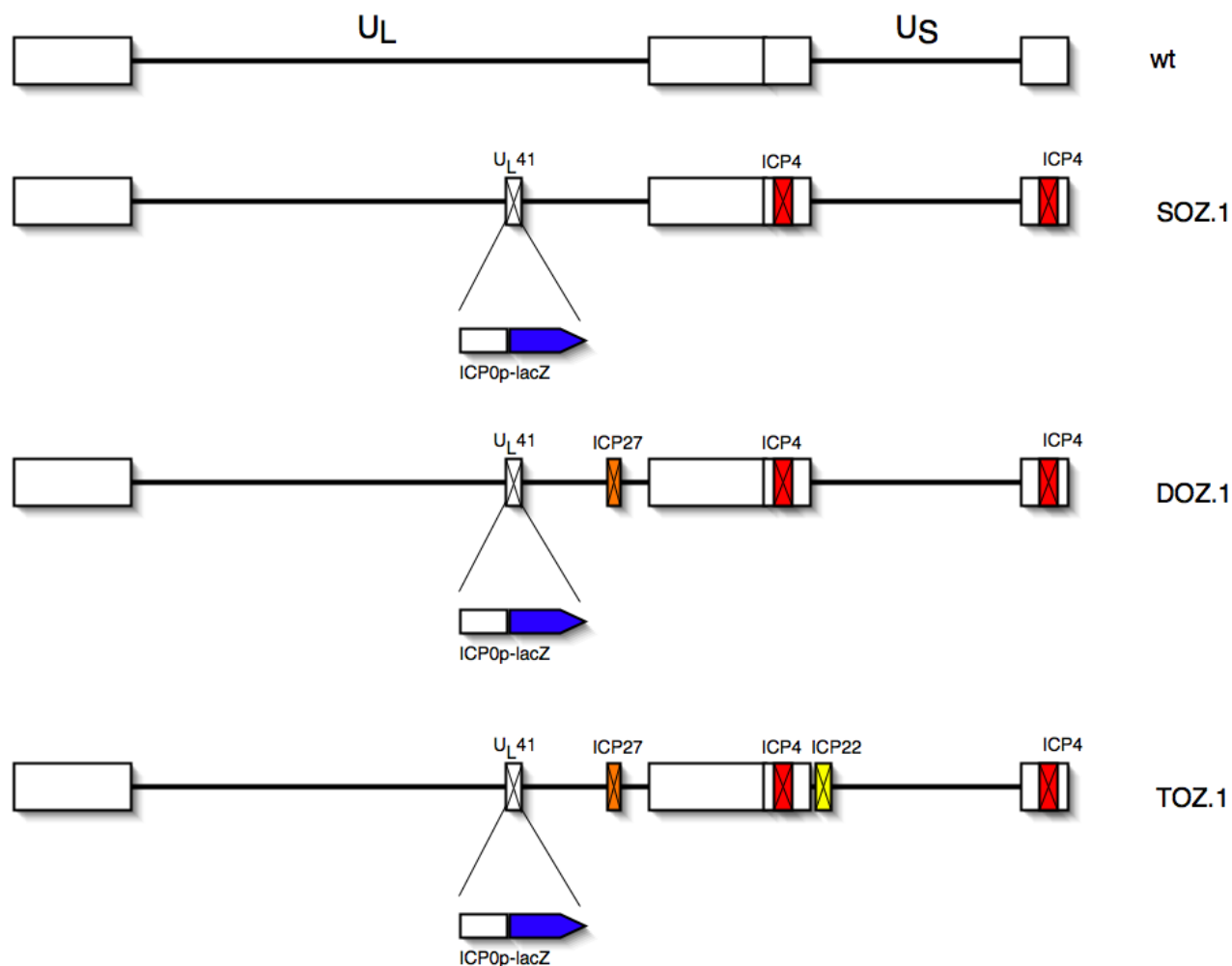


Figure 1: HSV vectors with IE gene deletions. (wt: wild type viral genome; SOZ: virus with single IE gene (ICP4) deletion; DOZ: virus with double IE gene (ICP4 and ICP27) deletions; TOZ: virus with triple IE gene (ICP4, ICP22 and ICP27) deletions.)

3.2. Complementing with Essential IE Genes

Replication defective viral vectors must be propagated on cells that provide essential IE gene products. Complementing cell lines providing ICP4 and/or ICP27 have been established in this lab and by others (Krisky et al.). One of the obstacles during the establishment of complementing cell lines is that some viral proteins (especially IE proteins) are toxic to the cells. Thus, inducible

expression of the complementing proteins is required. Unlike other inducible packaging systems that usually involves TetON/OFF system like the one in retroviral vector packaging systems, this inducible expression system takes the advantage of the natural responsiveness of the IE promoters (e.g. ICP4 and ICP0 promoters) to the viral tegument protein VP16. The expression plasmid containing the minimal IE promoter and the complementing protein is transfected into Vero (or another complementing cell line). In routinely maintained uninfected cells, the complementing protein expression is shut off. Upon infection, the VP16 in the virion turns the complementing protein expression on. For better growth of some vectors deficient for some unessential genes, these genes are sometimes transfected into the cells. Furthermore, to reduce the possibility of recombinational rescue of the defective virus, the deletion in the virus should be larger than the gene fragment transfected into the cell. By careful design of the complementing plasmid and the vector, the possibility of producing replication competent virions (RCVs) can be minimized (Krisky et al.).

3.3. The Conflict between Toxicity and Expression

The major source of toxicity in an ICP4, ICP22 and ICP27 deleted vector comes from ICP0 and ICP0 has pivotal roles in regulating global expression of the viral genome. Thus, here comes a conflict: it is favorable to remove ICP0 from the vector for its toxicity; however, when ICP0 is removed, expression from the vector is greatly impaired and the growth of the vector deteriorates as well.

There are several possible methods to address this issue. One is to introduce mutation in the ICP0 coding region that abolishes toxicity but retains its biological functions. One study showed that a single amino acid change could render the mutant virus non-toxic to neurons while the

growth of virus was not affected (Van Sant et al. 1999). It is not clear whether the gene expression from the virus was affected. Another concern is that this virus was ICP4, ICP22 and ICP27 positive, and thus it probably would not represent triple-deleted vectors. It will be a painstaking work to find other mutations in the coding region that will fulfill the goal.

Another approach is to reduce the ICP0 level to one where toxicity is minimized but the expression is retained. Hobbs et al showed that low level of ICP0 expressed from adenoviral vector was sufficient to activate viral genome (Hobbs et al. 2001). In the HSV genome, this approach may be realized by mutations in the promoter region. To test this method, ICP0 deletion mutations were constructed to drive lacZ expression in a triple-deletion viral backbone. Infection experiments showed that expression of lacZ was still high unless the deletion was relatively large. To verify if the deletions would work as expected, viruses with the mutant promoter driving ICP0 were constructed. While constructing these viruses, it was a surprise to find out that a replacement of the 5'-UTR by a segment of multiple cloning sites sequence reduced the ICP0 expression beyond detection while expression of transgenes in the virus stayed relatively high.

4. Materials and Methods

4.1. Plasmids

Plasmids pICP0d1~d6 were created by inverted PCR using corresponding primer pairs in Table 2 using plasmid pICP0p as template and then self-ligation. (Plasmid pICP0p is a pBluescript II SK vector with the Stu I/Drd I fragment of the ICP0 promoter inserted in its EcoR V site.)

Table 2: Primers used to generate deletions.

pICP0** constructs	Primer pairs	Sequences
pICP0d1	ICP0 U1	AAA CGC GTA ACC ACT CCC CTG G
	ICP0 L1	AAA CTA CCG GGA AGC GGA ACA AG
pICP0d2	ICP0 U2	AAA CCC GCA TTG GTC CCC TG
	ICP0 L2	AAA CGC GGG AGG CGG GGA ATA CC
pICP0d3	ICP0 U3	AAA CAG GCC AAG CCC CTG TTG C
	ICP0 L3	AAA CGC GAG GGG CCG TGT GTT C
pICP0d4	ICP0 U4	AAA CGG GTT GGG CCC CCA AAT CG
	ICP0 L4	AAA CGC TTC CCG CCT TCC CGA AG
pICP0d5	ICP0 U5	AAA CGC CTG CCT CCC CTG GGA C
	ICP0 L5	AAA CCG GTG TCC CCC AAA GAA CC
pICP0d6	ICP0 U6	AAA CGT CAC TGC CGC CCC TTT GG
	ICP0 L6	AAA CCC GGC GCC CCC AAA GA

Plasmid pUL41dKdSXba was constructed from plasmid pBS/UL41H-N (a pBlueScript vector (Stratagene) with UL41 HinD III/Not I fragment inserted in the EcoR V site) by sequentially removing Xho I and Sal I sites, removing a 600 bp fragment between two Sma I sites, removing

two Kpn I sites (and addition of an SnaB I site as the result of fill-in) and changing Sma I site to Xba I site by inserting an Xba I linker.

Plasmid pUL41p was constructed by deletion of a 600 bp Sma I/SmaI fragment of the pBS/UL41H-N and then insertion a Pme I linker in the Sma I site.

Plasmid pBSSKlacZXba was constructed as following: the 4.5kb Pst I fragment of pCMV β (Clontech) was inserted into Pst I site of pSP72 (Promega) to create p72-CMV; after checking the insertion directions, one clone with its lacZ in the orientation of EcoR I \rightarrow Xho I was treated with Sma I to create p72-CMVdP; the EcoR I –Pst I fragment of p72-CMVdP was inserted into EcoR I/Pst I sites of pBSSKdRVPme, which derived from pBluescript II SK by inserting a Pme I linker in EcoR V site, to create pBSSKLacZ(RI \rightarrow Pst); the Apa I site of pBSSKLacZ(RI \rightarrow Pst) was then changed to a Xba I site to create pBSSKLacZXba.

Plasmid p41LacZ was constructed by inserting the Xba I fragment of pBSSKLacZXba in Xba I site of pUL41dKdSXba with the LacZ direction of SnaB I \rightarrow Not I.

Plasmids p41ZOP-wt, -d1~d6 was constructed by inserting the EcoR I/Xho I fragments of corresponding pICP0-wt, -d1~d6, plasmids in EcoR I/Xho I sites of p41LacZ. Plasmid p41LacZ was also name as p41ZOP Φ as the promoterless control.

Plasmids p41ZOP-2T~5T was constructed by removing the Pme I/Xho I fragment of p41ZOPd2, p41ZOPd3, p41ZOPd4, p41ZOPd5, respectively.

pICP0 is a pBSSK derived plasmid containing the Stu I-Drd I fragment of the ICP0 promoter region in its EcoR V site. The Asp718-EcoR I fragment of pICP0 was cloned into Asp718/EcoR I sites of pSP72 to make p72-ICP0p. The BamH I-Bgl II fragment of p72-ICP0p was then inserted into Bgl II site of plasmid 28B(E3) to make p28-ICP0p. 28B(E3) is a pBlueScript-derived plasmid. 28B(E3) contains the Dra I (Sma I, sites in the parenthesis mean a site change)-

Stu I (Bgl II) fragment upstream ICP0 gene and Hpa I (Bgl II)-Dra I (EcoR V) fragment downstream ICP0 gene. In addition to the two viral genome fragments, 28B(E3) also contains a green fluorescence protein (GFP) gene flanked by Bgl II sites. After selection of clones with correct inserting direction, a Bgl II flanked fragment (from plasmid GFPOB) covering the Nco I-Hpa I region of ICP0 coding sequence was inserted into the Bgl II site of p28-ICP0p to yield p28-ICP0p-0.

4.2. Cell Cultures and Virus

Vero and complementing cell lines were maintained in DMEM complemented with 10% fetal bovine serum (Invitrogen) and 100 ug/ml penicillin and streptomycin at 37°C with 5% CO₂.

Recombinant viruses were created by infection-transfection as previously described with minor modifications. Briefly, cells were plated 12-24 hours before transfection at the density that at the time for transfection, the monolayer would be at ~80% confluence. The monolayer cells were first infected at MOI 0.01-0.1 in 1ml fresh DMEM with 10% FBS (serum media) with constant shaking for 1hour. Transfections were carried out with LipofectAmine2000 (Invitrogen) according to the manufacturer's instruction. Briefly, 30 minutes after the beginning of the infection, 1ug of plasmid was diluted in 400ul serum-free DMEM (SFM). In a separate tube, 4ul LipofectAmine2000 reagent was diluted in 400ul SFM. After 5 minutes incubation at room temperature, DNA and LipofectAmine2000 were combined and incubated at room temperature for an additional 30 minutes and were added to SFM washed infected cells. Cells were incubated at 37°C for 5 hours. 2 ml fresh SM was added to the cells after the incubation. The media was changed to fresh SM after overnight incubation.

Cells were checked under microscope daily. Cells were harvested after 2-3 days. Cells were broken up by one round of freeze-thaw and then by sonication. Cell debris was removed by centrifuge and the supernatant was frozen at -80°C until further screening for recombinant viruses.

Viruses were propagated on corresponding complementing cell lines. Virus stocks were produced as previously described (Krisky et al. 1997). Briefly, infected cells were collected and spun at 3000 rpm in a swing bucket centrifuge. Supernatants were reserved and pellets were subject to one round of freeze-thaw and sonication. Cell debris was spun out and the supernatants were combined. Viral particles were collected by spinning the supernatants at 20000 rpm for at least 30 minutes at 4°C. After centrifuging, supernatants were aspirated and a small amount of fresh media was add to cover the virus pellet. The pellets were kept on ice for 30 minutes and were resuspended in fresh media, aliquoted and frozen at -80°C until titration and other uses.

4.3. Viral Purification by Chromatography

Viral stocks could be further purified by chromatography to remove most of contaminants. Briefly, the viral pellet from high speed centrifuging was re-suspended in 1×PBS and kept at 4 while preparing the ion exchange column. HighTrap™ cation exchange column (Amersham) was sequentially washed with 5 column volumes of 0.5N NaOH, ddH₂O, 1×PBS, 1M NaCl and 7 column volumes of 1×PBS at the rate of 0.3ml/min. Viral suspension was then loaded onto the column at 0.3ml/min. The column was washed with 7 column volumes of 1×PBS and eluted with 0.45M NaCl. Glycerol was added to the eluate to a final concentration of 10% and the stock was aliquoted and stored at -80°C.

To determine the plaque forming units per ml, virus stock was subject to a serial of 10-fold dilutions and a portion was used to infect 1×10^6 corresponding complementing cells. After 1 hour of shaking at 37°C, cells were plated in 6-well plates. On the next day, the media was replaced with cell culture media containing 1% cellulose. After the plaques were visible, the cellulose media overlay was removed and the plaques were visualized by crystal violet staining.

In some cases, transducing unit (TU) was used. TU was determined by FDG staining or by GFP fluorescence with flow cytometry. For FDG staining, briefly, infected cells were collected 12-16 hours post infection (hpi) and resuspended in 1×PBS/1% FBS and incubated at 37°C for 5 minutes. Equal volume of 2mM FDG was added to the cell suspensions and cells were incubated at 37°C for exact 1 minute. Cells were then washed twice with ice cold 1×PBS/1% FBS and resuspended in 1×PBS/1% FBS. Analysis was carried out on a flow cytometer. For GFP fluorescence, infected cells were collected and analyzed directly.

4.4. Viral DNA Extraction

Viral DNA was extracted as previously described with a few modifications (Krisky et al. 1997). Briefly, infected cells were harvested and collected by centrifuging. Supernatant was removed and lysis buffer (10mM Tris-HCl, pH 7.4, 10mM EDTA, pH 8.0, 0.6% SDS) and proteinase K were added to the cell pellets. Lysis was carried out overnight at 37°C or 50°C with constant shaking. The lysate was then extracted once by equal volume of phenol-chloroform and then once with chloroform. The DNA was then precipitated with twice the volume of isopropanol and the DNA was spooled out with a sealed pasture pipet, washed in 70% ethanol and air dried. DNA was dissolved in TE and stored at 4°C for future use.

4.5. Polymerase Chain Reaction (PCR)

PCR were performed in a 50ul system which contains 1ul viral DNA, 10pmol of each primer, 5% DMSO, 0.2mM of each dNTP, 1× reaction buffer, 1mM MgCl₂ (for Promega Taq polymerase) or 2mM MgSO₄ (for Platinum High Fidelity Taq polymerase) and 2.5U of Taq polymerase (Promega) or 1.0U of Platinum High Fidelity Taq polymerase (Invitrogen). Reactions were carried out with incubation at 95°C for 5 minutes and then 30 cycles of 45 seconds at 95°C, 45 seconds at 55°C (or other temperatures determined by primer pairs) and 45 seconds at 72°C (69°C for Platinum High Fidelity Taq Polymerase) and a final incubation at 72°C for 5 minutes.

Primers used:

PU2: 5'-GGA GAG ACG ATG GCA GGA GC-3'

L2396: 5'-GGT TCC AGT GTA AGG GTC G-3'

56-Dave: 5'-GGC CCC TCG TTC CTA CCA GA-3'

Us2-Dave: 5'-GCG TCC GTG TTG TGC GTG TA-3'

4.6. Cell Survival Assay

Vero cells in suspension were infected at high MOI (10) at 37°C for 1 hour with shaking, centrifuged, washed with fresh media and plated in plates. At 2 days post infection (dpi) and 4 dpi, cells were collected and live cells were counted with trypan blue staining.

4.7. Colony Forming Assay

1X10⁶ Vero cells in suspension was infected at indicated MOI and shaken at 37°C for 1 hour. After the adsorption, the cells were subject to a serial dilution and plated in 6-well plates. The

cells were overlaid with culture media containing 1% methyl cellulose and maintained for 10 days or until the colonies were large enough to see by naked eye. The media was removed and the colonies were stained with crystal violet solution.

4.8. β -galactocidase Reporter Gene Assay

Infected cells were collected at indicated time points and β -galactocidase activity assays were carried out with Galacto-Light PlusTM System (Applied Biosystems) following the manufacturer's instructions. Protein concentrations of the cell lysates were measured with CBQCA Protein Quantitation Kit (Molecular Probes).

4.9. Southern Blots

Southern blots were carried out following previously described methods (Krisky et al. 1997). Briefly, viral DNA was digested with appropriate enzyme and resolved on 1% 0.5×TBE agarose gel. The gel was subject to sequential treatment of depurination (0.25M HCl, 30 minutes with shaking at room temperature), denaturing (1.5M NaCl, 0.5M NaOH, two times of 20 minutes each with shaking at room temperature) and neutralization (1.5M NaCl, 0.5M Tris-HCl, pH 7.0, two times of 20 minutes each with shaking at room temperature). DNA was then transferred onto a nylon membrane and UV-crosslinked. Hybridization was carried out with North2South Hybridization Kit (Pierce) according to manufacturer's instructions. Probes were prepared with North2South Random Primer Labeling Kit (Pierce).

4.10. Western Blots

Infected cells were lysed with Western blot lysis buffer (0.15M NaCl, 5mM EDTA, pH 8.0, 1% Triton X-100, 10mM Tris-HCl pH 7.4) with proteinase inhibitors. 10ug of total protein was resolved with 4-12% Bis-Tris PAGE (NuPAGE, Invitrogen) gel and transferred onto a PVDF membrane. The membrane was blocked with 5% milk in washing buffer (TBST, 50mM Tris-HCl, pH8.0, 150mM NaCl, 1mM EDTA, pH 8.0, 0.1% Tween 20) for 30 minutes at room temperature. Primary antibody was diluted in 5% milk and applied to the membrane overnight with shaking at 4°C. The membrane was washed three times with washing buffer and secondary antibody was applied in 5% milk in washing buffer for 1 hour at room temperature with shaking. Unbound secondary antibody was washed off with four 15-minute washing. Visualization was carried out according to the secondary antibody used. For HRP-conjugated antibody, the membrane was visualized with SuperSignal West Pico Chemiluminescence substrates (Pierce) following the instruction. For AP-conjugated secondary antibody, the membrane was developed with NBT/BCIP substrates (Sigma).

4.11. Real-time Quantitative PCR

Total RNA was prepared using RNeasy RNA Extraction Kit (Qiagen). 800ng of total RNA was reverse-transcribed with SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions using random hexamer primers. 10% of the reverse transcription reaction was used as the template for real-time quantitative PCR using TaqMan real-time quantitative PCR system (Applied Biosystems) following its instructions. A 100X dilution of the reverse transcription reaction was used to detect 18S RNA as the control.

To calculate the relative amount of mRNA, the comparative critical threshold (CT) method was used. Refer to [ABI Prism 7700 Sequence Detection System User Bulletin #2](#) for the details of the calculation.

Primers and probe used in the ICP0 real-time quantitative PCR:

Forward: 5'-AAC GCC AAG CTG GTG TAC CT-3'

Reverse: 5'-TCA CGA TCG GGA TGG TGC-3'

Probe: 5'-TGA CGC CCA GCG GGT CGT TC-3'

5. Results

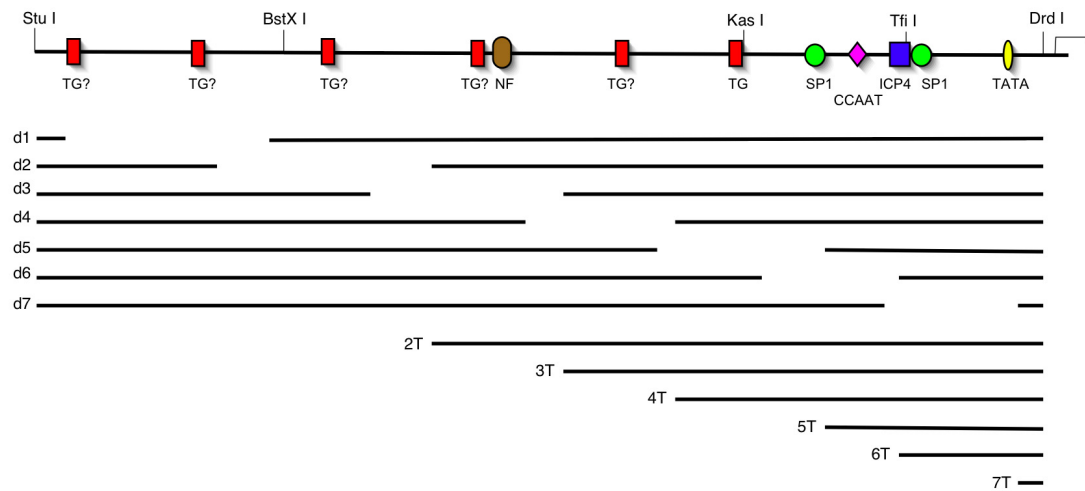
5.1. Reporter Gene Assay

ICP0 promoter is one of the most complicated promoters in HSV genome, containing several VP16-corresponding sequences, Sp1 motifs as well as other potential *cis*-signals (Figure 2(a)). Deletions in the promoter region were created by inverted-PCR in plasmids. To test whether these deletion mutations could reduce the expression of ICP0, mutated promoters were incorporated into the lacZ expression cassette in an ICP4, ICP22 and ICP27 negative viral backbone (Figure 2(b)) and β -gal activities at different times were assayed from lysates of infected Vero cells (MOI 2).

At 6 hours post infection (hpi), adequate amount of β -gal could be readily measured (Figure 3) and at 24 hpi, β -gal accumulation reached its plateau, disregarding constructs (data not shown). At 6 hpi, full length ICP0 promoter (wt) efficiently drove the expression of lacZ. At this time point, all the internal deletion mutants tested (d1~d6) and some 5' truncation mutants (2t and 4t) could initiate the expression of the reporter gene but at a lowered level (10~60%). Other mutants, as well as the promoterless construct N, were unable to drive efficient expression of LacZ. At early hours post infection, the reporter gene activity mainly reflected the capability of the promoter mutant initiating transcription. Deleting individual elements did affect the expression level but could not eliminate the expression. This indicated that some of these elements seemed be able to compensate for each other. In other words, the promoter possesses redundant elements as for the VP16 transactivation. One internal deletion, d6, showed a much lowered β -gal level, which implied that the Sp1 site deleted in that construct might play important roles in

constitutive expression. The largest drop of expression occurred between 2T and 3T, which indicated that the uppermost four TAATGARAT motifs are important for the VP16 transactivation.

(a)



(b)

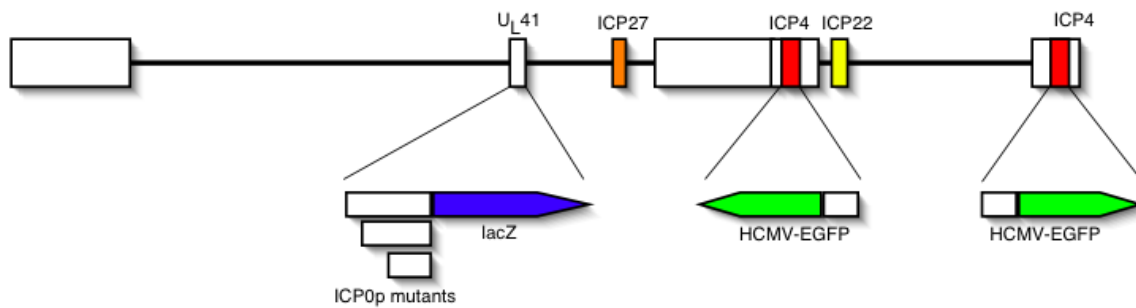


Figure 2: (a) Diagram of the promoter *cis*-elements and deletions: TG, TAATGARAT; NF, NFκ-1 like binding motif; Sp1, SP1 binding motif; ICP4, ICP4 binding motif; (b) Reporter gene assay constructs: HCMV, HCMV IE promoter.

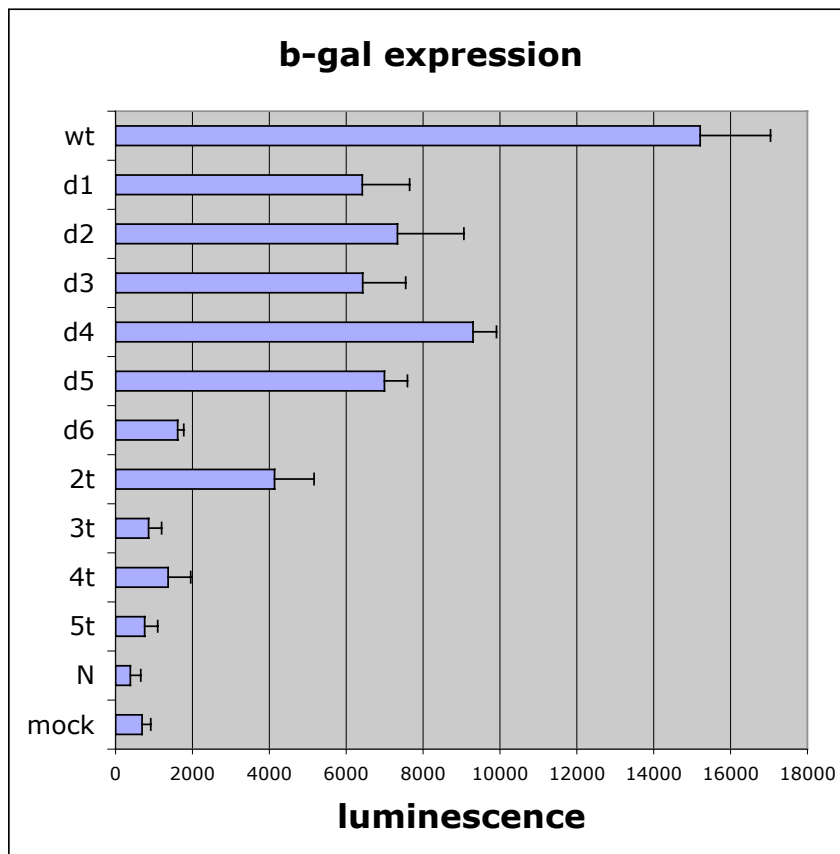


Figure 3: β -gal assay at 6 hours post infection with reporter gene assay constructs.

5.2. Construction and Confirmation of JDTOZHERO

5.2.1. Construction of JDTOZHERO

Reporter gene assays did show that deletions in the promoter could reduce the expression. However, the optimal level at which the balance between toxicity and expression might be reached was not determined. Thus, a new set of viruses in which ICP0 was driven by deletion mutant promoters was then constructed.

To construct these viruses, the ICP0 locus in JDQOZ was restored with plasmids covering the ICP0 region. JDQOZ is a virus with multiple modifications compared to wild type virus:

deletions in genes for ICP0, ICP4, ICP22 and ICP27; a deletion spanning the internal inverted repeat sequences (IRL and IRS, referred here as joint and thus this deletion is referred as joint-deletion); and an insertion of ICP0p-lacZ cassette in the ICP0 (Figure 8).

JDTOZHERO was constructed as the control virus for these viruses. The plasmid used to restore ICP0 gene in JDTOZHERO is p28-ICP0p-0, which contains viral genomic fragments of Dra I-Drd I upstream and Nco I-Dra I downstream of ICP0 gene, respectively. For details of construction of plasmid p28-ICP0p-0, see Materials and Methods. After transfection with p28-ICP0p-0 and subsequently infection with JDQOZ in 433 cells (complementing ICP4, ICP27 and ICP22), recombinant clones were selected and purified by three rounds of single-plaque purification confirmed by PCRs with primers (L2396 and PU2, data not shown) within ICP0 gene. Then the HCMV-EGFP expression cassette was incorporated into the ICP4 locus with the same transfection-infection method.

5.2.2. Southern Blots

Southern blots were performed to verify the genome structure of JDTOZHERO. Viral DNA was digested with either Dra I (for ICP4 loci) or Sac I or Nco I (both for ICP0 loci) and processed as mentioned in Materials and Methods. Blots demonstrated that the genome of TOZHE has two copies of ICP0 (Figure 5) and ICP4 (Figure 6) while JDTOZHE and JDTOZHERO only have one copy. The other copy located in the joint region has been deleted. (Refer to Figure 4 for the locations of the digestion sites and probes.)

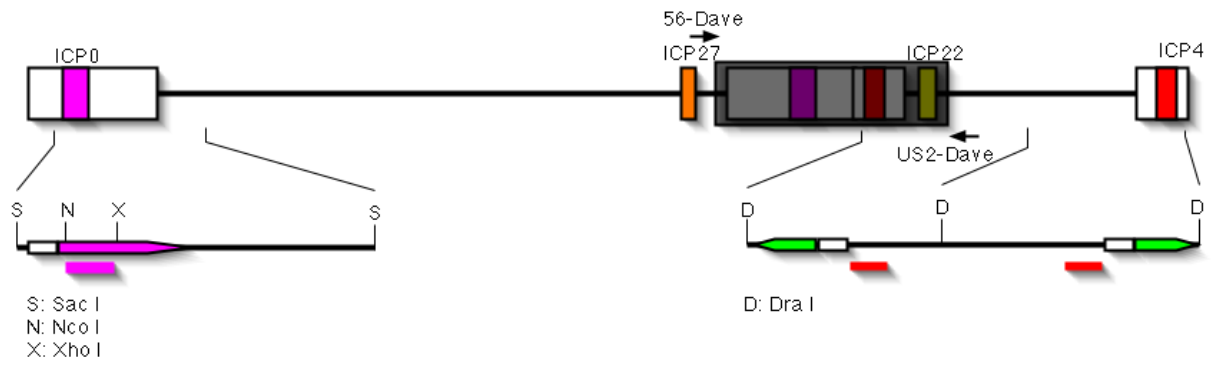


Figure 4: Diagram of Southern blots and joint-region PCR primers of JDTOZHERO.

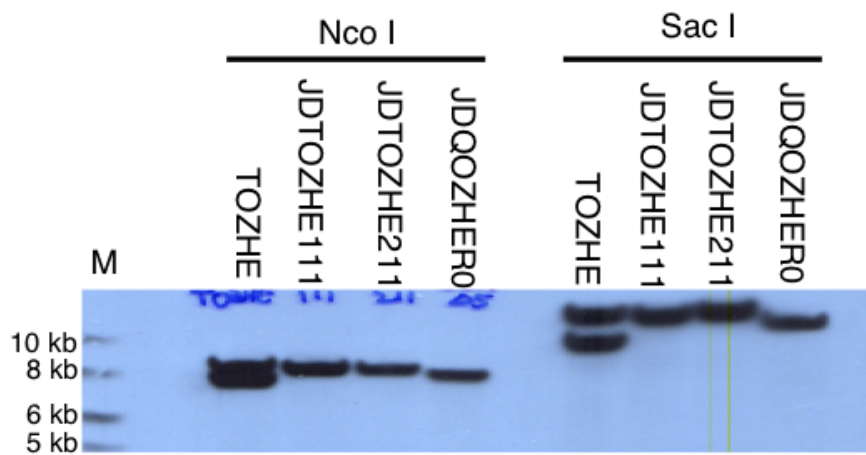


Figure 5: Southern blot of ICP0 locus with either Nco I (left) or Sac I (right) digestion.

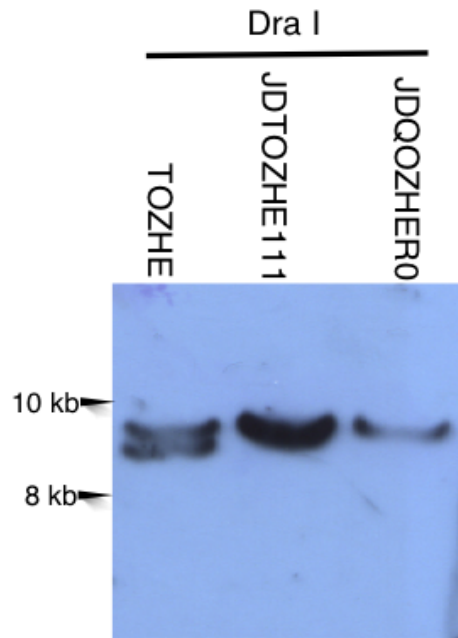


Figure 6: Southern blot for ICP4 locus with Dra I digestion.

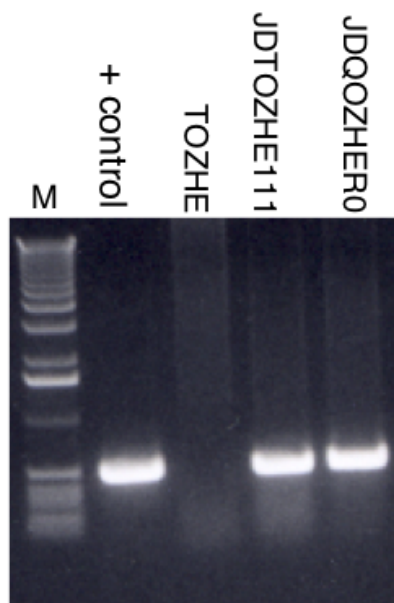


Figure 7: PCR at joint region.

5.2.3. PCR Results

PCRs were performed with corresponding primers to verify the modifications in the viral genome. Primers located in U_L56 (56-Dave) and U_S2 (US2-Dave) were used to amplify the joint region. Refer to Figure 4 for the locations of the primers. Under experimental PCR conditions, the fragment between these two primers in TOZHE is too large (15 kb) and could not be amplified while in joint-deleted viruses, i.e. JDTOZHE and JDTOZHERO, the fragment (500 base pairs) could be amplified (Figure 7).

5.2.4. Sequencing Results

Sequencing at the restored ICP0 gene locus revealed an unforeseen modification in the gene. The sequences from Drd I to Nco I was replaced by a section of multiple cloning sites sequences from some cloning vector. This happened during the cloning step from plasmid p28-ICP0p to plasmid p28-ICP0p-0 because the segment used to restore ICP0 coding sequences (Bgl II fragment from plasmid GFPOB) does not have the missing sequences (see Materials and Methods). This modification removed the natural transcription start site as well as the entire 5' untranslated region (UTR) of ICP0 gene but the TATA box is reserved. The natural translation start site is also reserved. The sequences of the wild type ICP0 locus and the mutated are compared below.

Sequence at wild type ICP0 locus: (TATA box, transcription starting site and translation starting site are marked. Replaced sequences are highlighted.)

```
GGGG[TATAA]GTTAGCCCTGGCCCGAC[AGTCTG+GTCGCATTTGCACCTCGGCACTCG  
GAGCGAGACGCAGCAGCCAGGCAGACTCGGGCCGCCCTCTCCGCATCACCACAG  
AAGCCCCGCCTACGTTGCGACCCCCAGGGACCCTCCGTCCGCGACCCTCCAGCCGCA  
TACGAC]CCCC[ATG]GAGCCCCGCCCGGAGCGAGTACCCGCCGG
```

Sequence at the same locus in JDTOZHERO:

```
GGGG[TATAA]GTTAGCCCTGGCCCGAC[GGATCGAATTCATCGATGATATCAGATCTC
```

GAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCCATGGAGCCCCGC
CCCGGAGCGAGTACCCGCCGG

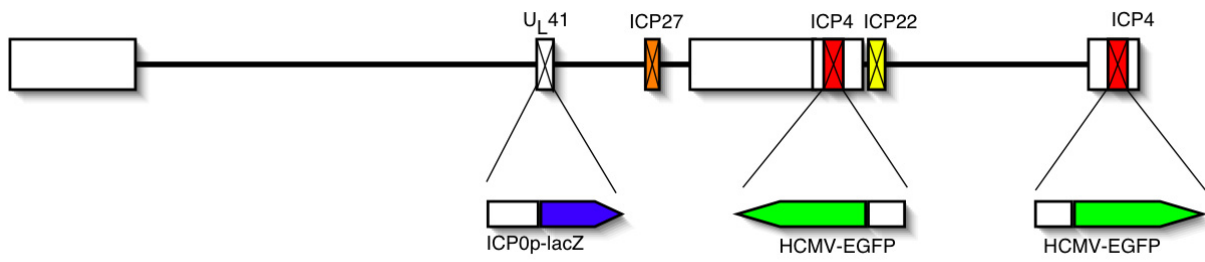
5.3. Characterization of JDTOZHERO

Along construction of JDTOZHERO, it was noticed that the virus possesses some unexpected features, which were first noticed when compared to JDTOZHE. JDTOZHERO was projected to behave the same as JDTOZHE: they both are joint-deleted thus they only have one copy of ICP0 gene in their genome; they both are ICP4, ICP22 and ICP27 defective; they both have HCMV-EGFP in ICP4 locus and ICP0-lacZ in U_L41. Thus, a set of experiments was carried out to further characterize the virus. The virus was tested against TOZHE (two copies of ICP0), JDTOZHE (one copy of ICP0) and JDQOZHE (ICP0 null) at the same time. The modifications in these viruses are listed in Table 3. Also, refer to Figure 8.

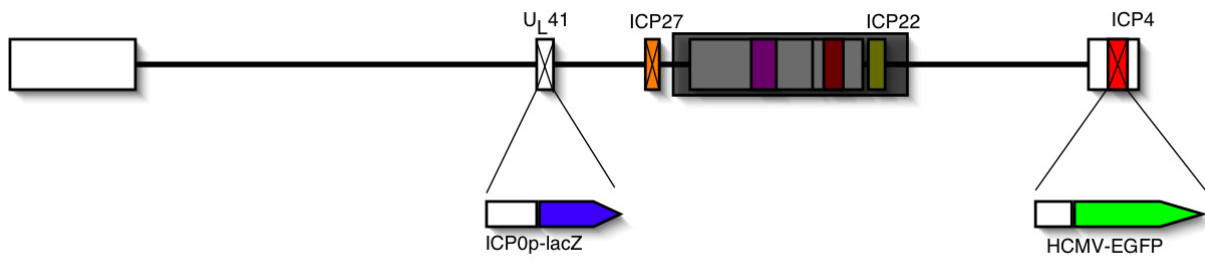
Table 3: Viruses used in the experiments.

Modifications	TOZHE	JDTOZHE	JDTOZHERO	JDQOZHE
Joint deletion	No	Yes	Yes	Yes
ICP4	-	-	-	-
ICP0	+ (2 copies)	+ (1 copy)	+ (1 copy)	-
ICP22	-	-	-	-
ICP27	-	-	-	-
HCMV-EGFP	+ in ICP4 loci	+ in ICP4 locus	+ in ICP4 locus	+ in ICP0 locus
ICP0-lacZ in UL41	+	+	+	+

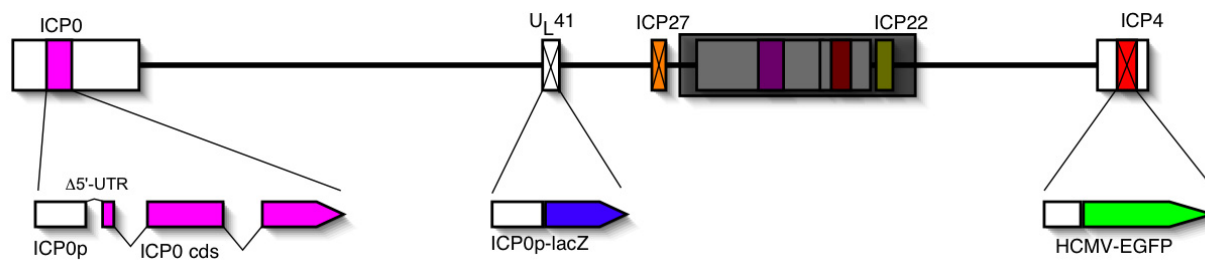
(a)



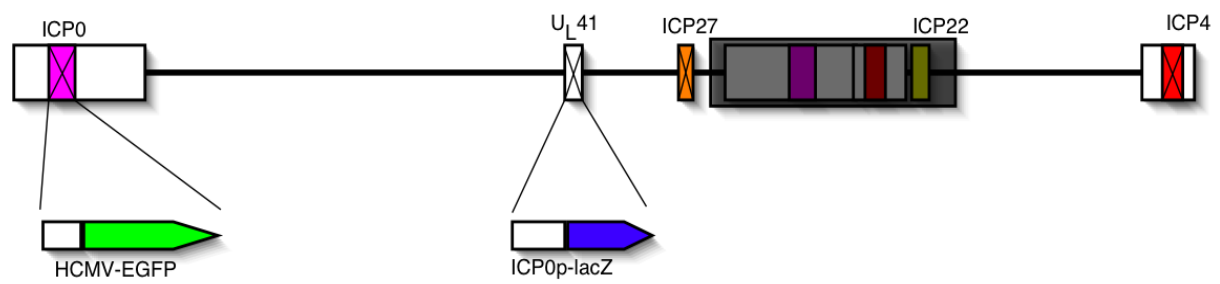
(b)



(c)



(d)



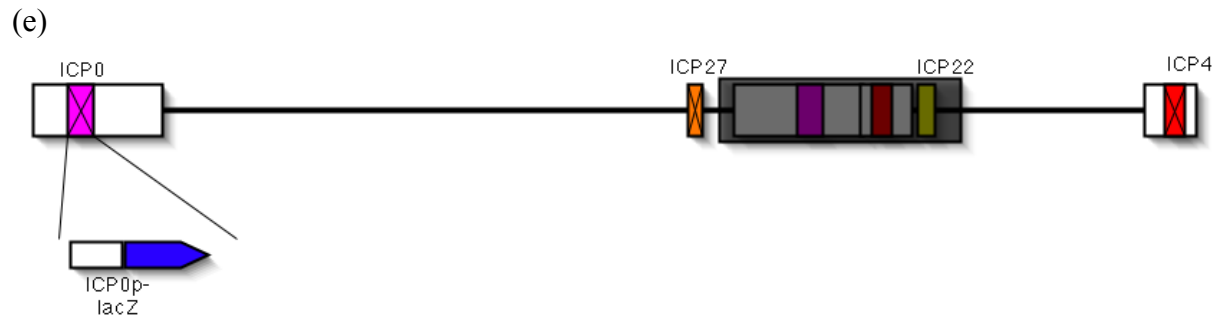


Figure 8: Constructs used in the study. (a) TOZHE; (b) JDTOZHE; (c) JDTOZHERO; (d) JDQOZHE; (e) JDQOZ

5.3.1. Viral Transduction of Cells

Vero cells were infected with these four viruses at the MOI of 3 and 10 using TU (assayed on U2OS, see Material and Methods). At 24 hours post infection (hpi), cells were trypsinized and collected. The cell suspensions were subject to flow cytometry to measure the GFP signal. At MOI of 10, nearly 80% of total cells infected with TOZHE or JDTOZHE were transduced, marked as GFP positive, while about 50% of total cells infected with JDTOZHERO were positive. The mean intensity of GFP signal of the positive cells in TOZHE or JDTOZHE infected cells were about twice that in JDTOZHERO infected cells. While almost 30% cells infected with JDQOZHE were marked as positive, the mean intensity of the signal is only about 3% of that from TOZHE or JDTOZHE infected cells (Figure 9).

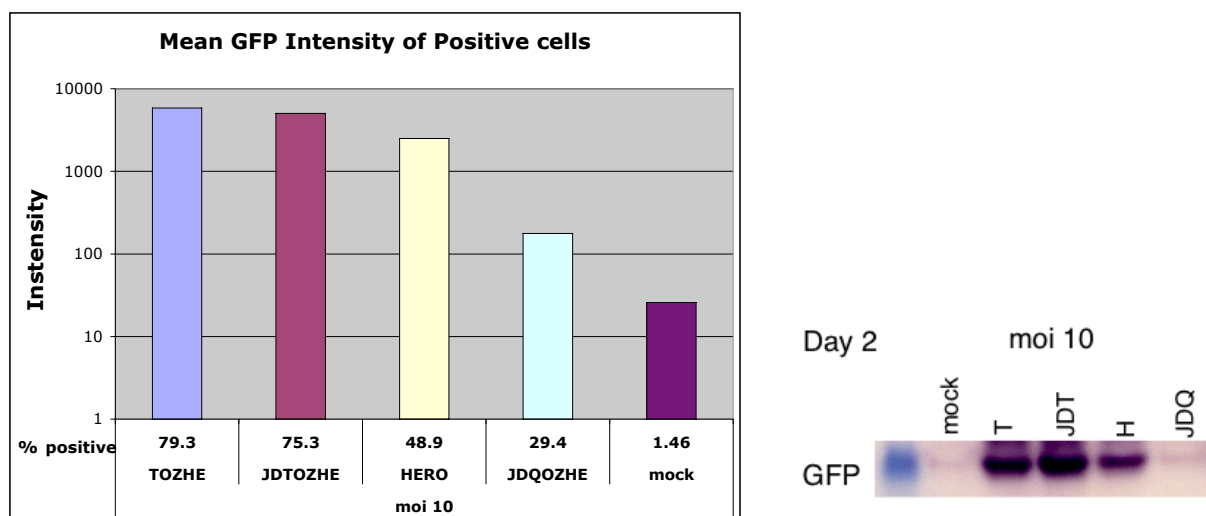


Figure 9: GFP signals measured by flow cytometry (left) and Western blot for GFP (right).

5.3.2. Transgene Expression

Infected Vero cells (MOI 3 or 10) were harvested and transgene expression was measured. β -gal level was measured using the Galacto-Light Plus Chemiluminescence Kit (Tropix). 2 days post infection, the β -gal activity from JDTOZHERO infected is about 50% of that from TOZHE or JDTOZHE infected cells. As expected, the activity level from JDQOZHE is very low, only about 1% of that from TOZHE (Figure 10).

The expression of another transgene in the genome, EGFP, was determined by western blot. Western blot is not a full quantitative measurement method but it clearly showed that at 2 days post infection, abundant amount of EGFP was expressed from JDTOZHERO infected cells. This amount is estimated to be about 50% of that from TOZHE or JDTOZHE infected cells and the amount of EGFP from JDQOZHE was beyond detection limit (Figure 9).

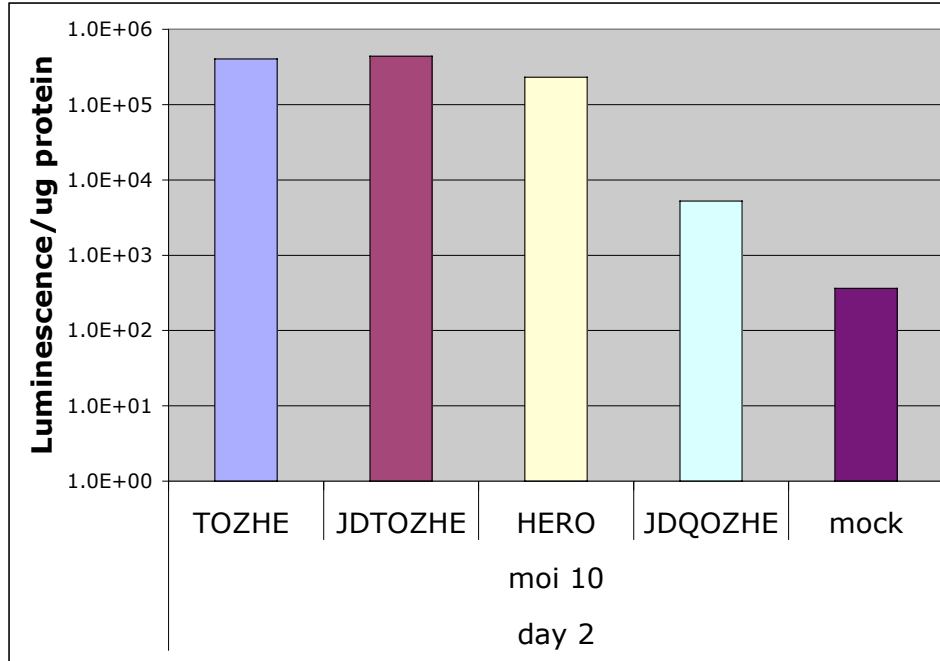


Figure 10: β -gal activities from viruses.

5.3.3. ICP0 Expression

As mentioned above, ICP0 has a great impact on the global expression from the viral genome. Thus, it is of great interest to investigate the ICP0 expression from JDTOZHERO since transgene expression from this virus lies between that from TOZHE and JDQOZHE. Western blots showed that ICP0 protein from TOZHE and JDTOZHE infected cells was in abundant amount that was easily detected while surprisingly, ICP0 was not detected from JDTOZHERO infected cells under experimental conditions (Figure 11).

ICP0 mRNA level was also measured using real-time quantitative RT-PCR. ICP0 mRNA signal was not detected from JDQOZHE or mock infected cells as expected (not shown). The ICP0 mRNA level from JDQOZHE is relatively the same as that from TOZHE (91-104%). The ICP0

mRNA level from JDTOZHERO infected cells was only about 8% (7-9%) of that from TOZHE infected cells (Figure 11).

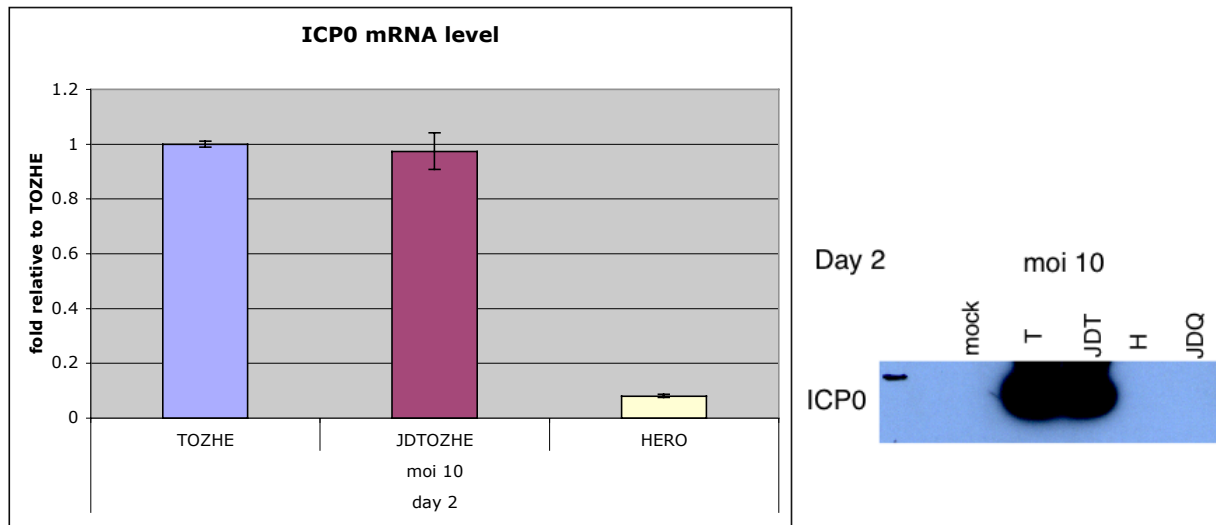


Figure 11: ICP0 quantitative RT-PCR and Western blot.

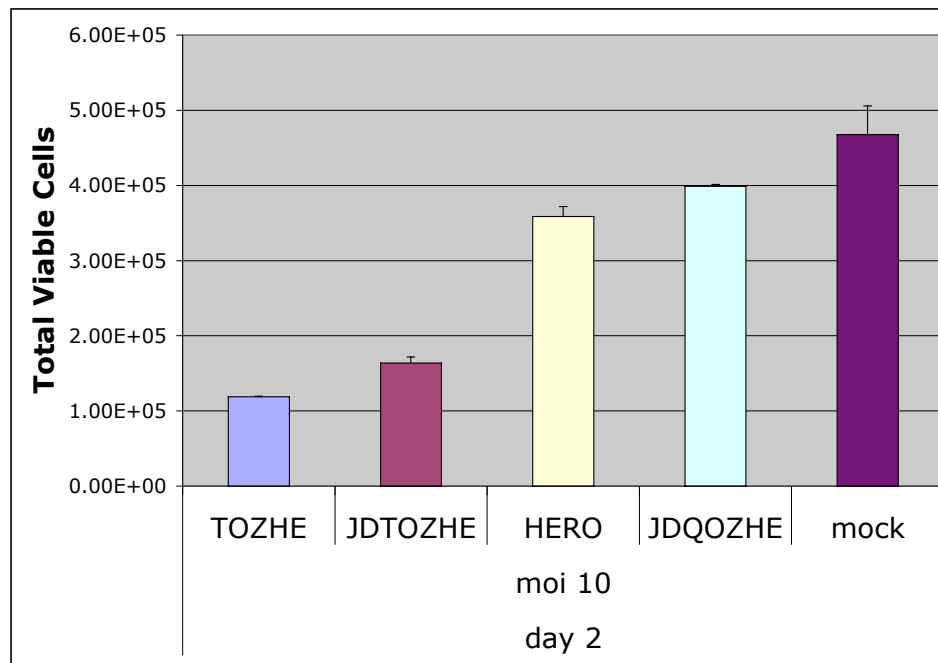
5.3.4. Cytotoxicity of JDTOZHERO

As mentioned in the introduction, in ICP4-, ICP22- and ICP27- background viruses, ICP0 is the major source of cytotoxicity in vitro. Since the level of ICP0 expression from JDTOZHERO is greatly reduced, it is of great interest to investigate the toxicity of the virus. Two similar methods, viable cell counting and colony forming assay, were used to measure the toxicity in vitro. Both methods measure the acute toxicity of the vectors.

Vero cells were infected at MOI of 3 or 10 and plated in 6-well plates. Cells were trypsinized after 48 hours and stained with trypan blue. Unstained cells are then counted in a hemacytometer. The viable cell number from JDTOZHERO infected cells was significant higher

(77.8% of mock infected, MOI 10) than TOZHE ($p < 0.01$, Student's t-test) or JDTOZHE ($p = 0.012$) (26.7% and 35.6% of mock infected, MOI 10, respectively) (Figure 12).

Vero cells were also infected at MOI of 10. After 1 hour of adsorption, the cells were subject to a series of dilutions and estimated 100 cells were plated in 6-well plates. Cells were overlaid with 1% methyl cellulose media. After the colonies were visible by naked eyes, the plates were stained with crystal violet and the colonies were counted. The results were similar to that of viable cell counting method. The colonies from JDTOZHERO infected cells were significantly more than from TOZHE ($p = 0.011$) infected cells (Figure 12).



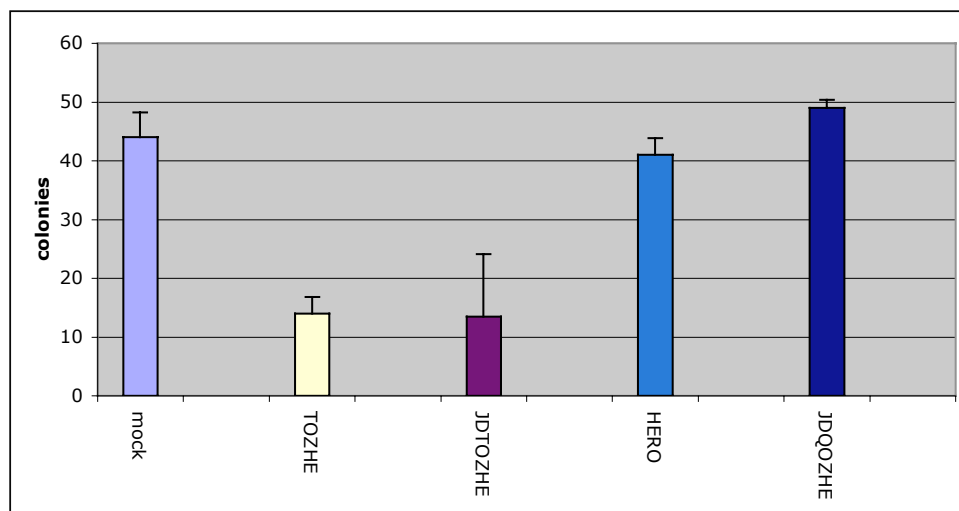


Figure 12: Cell survival (top) and colony forming assays (bottom). Mean of the number of colonies and range.

6. Discussion

HSV-based vectors are emerging as a promising vehicle in human therapeutic gene transfer. Possessing some advantages, HSV vectors also have some disadvantages. The major drawback of current vectors is the remaining toxicity. The purpose of the modifications on the viral genome is to eliminating the toxicity but retaining the high transduction efficiency, as well as keeping it production feasible. The production of HSV vectors and subsequent purification has been discussed elsewhere ([Wechuck, PhD dissertation, 2002](#); [Ozuer, PhD dissertation, 2002](#)).

The main purpose of this study was to investigate the regulation of ICP0 expression so that a construct with high in transduction efficiency and low in *in vitro* toxicity could be found.

Reporter gene assays were first performed to test whether any deletion mutations introduced into the promoter region could effectively change ICP0 expression. Deletion mutant promoters were used to drive the lacZ reporter gene. Data from these experiments did show that deletions in the promoter could reduce the expression of the reporter gene (0~60%) compared to the wide type promoter. However, the optimal ICP0 level at which the toxicity and gene expression reach the balance could not be determined.

To determine the balancing ICP0 level, these mutant promoters must be used to drive ICP0 protein itself. Thus, a new set of viruses was constructed. Virus JDTOZHERO was first constructed as the control virus for this purpose. However, this virus showed some unique features that are of great interest for further investigation.

The first unexpected factor of JDTOZHERO was that its transduction ability was between that of TOZHE and JDQOZHE, which possesses two copies of ICP0 gene and no ICP0, respectively.

Quantitative assay by reading the GFP signal by flow cytometry confirmed the observation. At MOI of 10, JDTOZHERO was able to transduce about 50% of cells while TOZHE was 80%. About 30% of the cells infected with JDQOZHE were marked as “positive”. This percentage was higher than expected since only occasional green cells can be observed under fluorescence microscope. However, the intensity of the signal in JDQOZHE infected cells was extremely low (about 3% of the intensity of TOZHE infected), which indicated that flow cytometry is a more sensitive detection method. The distribution of the signals in JDTOZHERO was shifted to lower end relative to that of TOZHE or JDTOZHE infected. The mean intensity of the positive signals, one of the parameters for the distribution, revealed that GFP expression was also lower in JDTOZHERO infected cells in addition to the lowered efficiency of transduction. However, GFP expression in JDTOZHERO was still strong, about 50% of the TOZHE infected cell intensity.

The MOI used in the study was based on the transducing unit (TU) assayed on U2OS cells. The reason to use TU instead of plaque forming unit (PFU) is that ICP0-null mutant form plaques poorly, even on ICP0-complementing cells. The ratio of TU to PFU (assayed on 433 cells, an ICP4, ICP27 and ICP22 complementing cell line) is about 10 for TOZHE and JDTOZHE and 150 for JDTOZHERO. There will be more infectious viral particles in JDTOZHERO than in TOZHE and JDTOZHE in the same amount of PFU. This might lead to misleading results like in the case of cyclin D1 (Everett 2004). An alternative method to measuring TU on U2OS cells is to titrate all viruses (by PFU) on an ICP0-complementing cell line.

It is of notice that at the experimental MOIs, the behavior of JDTOZHE (one copy of ICP0 gene) is indiscernible to that of TOZHE. This might mean that only one copy of ICP0 is sufficient for normal viral growth. The difference between these two may be distinguished at lower MOI.

The expression of the two transgenes in the construct was further examined. GFP protein was measured by Western blotting. Although Western blotting is not a fully quantitative method, the approximate expression level can be estimated from the blots. At 48 hours post infection, strong GFP immuno-blotting signals could be seen from TOZHE and JDTOZHE infected cells while moderate signal from JDTOZHERO. No GFP could be detected from JDQOZHE. This blotting result confirmed that obtained from flow cytometry. Expression of the other transgene, lacZ, is detected with the much more sensitive and fully quantitative chemiluminescence method. Nevertheless, the results were similar to that of GFP. At 48 hpi, while β -gal level from JDTOZHERO infected cells was not as high as that from TOZHE or JDTOZHE infected cells, it was much higher than that from JDQOZHE infected cells. These results were consistent with previous observations in the lab on ICP0-positive and ICP0-null viruses. The interesting thing is that under the experimental conditions (MOI of 10 and 3), the transgene expression from virus with one copy of ICP0 (JDTOZHE) was indistinguishable from that from viruses with two copies of ICP0 (TOZHE) and that transgene expression of JDTOZHERO sat between viruses with one copy of ICP0 and no ICP0. Thus, it is speculated that the ICP0 level of JDTOZHERO is lowered compared to that of JDTOZHE.

Surprisingly, ICP0 protein was not detected in JDTOZHERO infected cells by Western blotting while TOZHE and JDTOZHE infected cells gave out strong signals as expected. There are several possibilities why ICP0 protein was not detected: a) the amount of the protein is so low that it is beyond the detection limit of the method; and b) the sequence in the 5'-UTR produced a mutant protein that is not recognizable by the antiserum. The antiserum was raised against a segment in the carboxyl terminal of the protein. If the replacement in the 5'-UTR has changed the C-terminal, it is reasonable to reckon that the N-terminal is affected, too. However, it is very

unlikely that a totally changed ICP0 protein would still function the same way as its natural protein. However, the exact transcript has not been mapped and more experiments are required. Thus, lowered expression seems to be the explanation.

The detection limit of Western blotting has not been determined yet and there is no way to tell the approximate level of ICP0 protein from JDTOZHERO infected cell now. The lowered level of protein could be caused by reduced transcription, unstable mRNA, and/or reduced translation. ICP0 mRNA level was determined by TaqMan real-time quantitative PCR. The results showed that the mRNA level from JDTOZHERO infected cells is about 10% of that from TOZHE or JDTOZHE infected cells. However, it seemed that this could not explain the undetectable level of protein. Thus, reduced translation might be also involved. This method could not distinguish the cause of the low mRNA level. It has been reported that the multiple cloning site sequences in the 5'-UTR destabilize the mRNA and this could contribute to the result. However, the possibility of reduced transcription could not be excluded.

One issue related to ICP0 is toxicity. Since the most likely reason unable to detect ICP0 protein in JDTOZHERO infected cells was the expression level fell below the detection limit, it is naturally to suspect that the virus should be much less toxic. *in vitro* cytotoxicity assays confirmed this. The toxicity of JDTOZHERO is much lower than that of TOZHE or JDTOZHE and not significantly different from that of JDQOZHE. Thus, compared to TOZHE or JDTOZHE, JDTOZHERO should be a safer vector. The situation *in vivo* is a little different from that of *in vitro* experiments. A TOZ based vector was well tolerated in animal experiments and elicited no noticeable side effect. However, JDTOZHERO gives some more safety margins in places where the death of the cell could cause big problems, such as neurons.

7. Conclusion and Future Work

HSV vectors have become one of the most promising gene transfer vectors because of the virus's unique features. A great deal of efforts has been made in the modification of the viral genome, with the goal of a safe but efficient vector. For applications in which cell death should be avoided, the conflict between efficiency and safety has not been fully resolved. For vectors with multiple IE gene deletions, one potential solution to lower the toxicity from ICP0 is to reduce the ICP0 protein level to which the global viral expression is not fully repressed by the cellular mechanisms. For this purpose, lacZ reporter gene assays were first performed to investigate the possibility of lowering ICP0 expression with deletion mutations in the promoter region. The results showed that deletions in the promoter could reduce the expression to certain extent. To verify the results and to find out the optimal ICP0 level, another set of viruses were constructed. JDTOZHERO were constructed as the positive control virus for this purpose. However, JDTOZHERO showed some unique features that were worth further investigation. The results showed this construct was able to direct transgene expression efficiently, although the expression level was not as high as TOZHE or JDTOZHE. At the meantime, JDTOZHERO showed much lower toxicity *in vitro* compared to TOZHE or JDTOZHE. Although TOZHE has been shown non-toxic in animal experiments, the application of JDTOZHERO would give a further safety margin.

However, there are still some questions unanswered. One is that the exact cause of the lowered toxicity in JDTOZHERO. Currently, it is presumed this was caused by lowered level of ICP0 but

the possibility of a mutated ICP0 protein could not be excluded. The mapping of the ICP0 transcript in JDTOZHERO is required to answer this question.

Another question is that whether ICP0 expressed from JDTOZHERO, either at lowered level or as a mutant protein, would disrupt the cellular defense mechanisms. For example, does it disrupt ND10 and other cellular components; and is JDTOZHERO sensitive to IFN, (Mossman et al. 2000)? More experiments are needed to answer this question.

The ultimate question is whether this construct would function *in vivo*. To answer this question, animal experiments are needed. At the same time, genes with biological functions (e.g. factor IX) could be inserted into the construct and the new vector could be tested in the animals.

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